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Targeting of RNAs to ER Subdomains and its Relationship to Protein Localization

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Abstract The targeting of proteins to specific subcellular regions is directed by a variety of signal elements. Many of these signals consist of amino acid residues (peptide sorting signals) arranged contiguously or in a three-dimensional array. In addition to posttranslational processes, proteins can also be localized to specific regions of the cell by the targeting of their cognate RNA. Ongoing studies in developing rice endosperm have shown that the RNAs that code for the major storage proteins are localized to specific subdomains of the cortical endoplasmic reticulum (ER), and that there is a tight correlation between the initial site of RNA localization and the final destination of the encoded protein in the endomembrane system. The segregation of RNA onto distinct ER subdomains may be a necessary and sufficient step for the localization of the coded protein in the cell.

1 Introduction

The endoplasmic reticulum (ER) serves as the entry site for proteins that are to be secreted or located at one or more locations in the endomembrane system in eukaryotic cells. Proteins are targeted to the secretory system with the emergence of the signal peptide during initial protein synthesis, which is recognized by the signal recognition particle. The resulting translationally arrested complex (mRNA–ribosomes–translation factors) is then mobilized to the ER, which enables the translocation of the growing polypeptide chain to continue through the ER membrane to the lumen. Within this organelle the polypeptide is folded and assembled to a correct conformational maturation state aided by a plethora of resident molecular chaperones. The presence of additional peptide sorting signals or interacting domains may enable the protein to be either retained within the ER or transported to other destinations within the endomembrane system (Vitale and Denecke 1999; Vitale and Ceriotti 2004). In the latter instance, proteins can be exported from the ER by two distinct pathways, one involving the Golgi apparatus

and a second that is suggested to be Golgi-independent (Hara-Nishimura et al. 1998).

An ideal system for studying the biochemical and cellular events of ER-dependent translation is developing seeds. During seed development, one or more organs synthesize vast quantities of reserve protein, which assemble within the ER lumen itself or within protein storage vacuoles to form discrete organelles termed protein bodies. One question that immediately arises is why some plants utilize the ER lumen whereas others develop protein storage vacuoles. Although the exact cellular basis for this is not known, it is clear that the nature of the storage protein does not dictate its ultimate storage site. For example, members of the prolamine superfamily that share common structural domains are not stored in the same compartment. The 2S albumins of several dicotyledonous plants and prolamins of wheat and barley are transported to the protein storage vacuole, whereas the maize prolamins (zeins) assemble within the ER lumen to form intracisternal inclusions (Shewry et al. 1995). Although peptide sorting signals as well as peptide interacting domains are ultimately responsible for the final destination of proteins, evidence is beginning to emerge that the intracellular location of a protein may also be influenced by where it is being translated on the ER (Crofts et al. 2004). In this chapter we elaborate on this hypothesis by discussing recent advances in the analysis of the relationship between RNA targeting to specific ER subdomains and protein localization.

2 RNA Localization in Animals and the Role of the ER

RNA localization is recognized as an important process in controlling the synthesis of proteins at specific sites within the cell. More than 100 messenger RNAs are now known to be targeted in a wide variety of eukaryotic cells. This process is essential for cell fate determination in yeast (Chartrand et al. 2001), during early vertebrate development (Bashurullar et al. 1998; Palacios and Johnston 2001), in polar cell growth of somatic cells (Ainger et al. 1997; Carson et al. 1998; Shestakova et al. 2001), and in mediating cell motility (Kloc et al. 2002). The use of this mechanism for several different developmental and cellular processes suggests that the process of RNA localization is common to all eukaryotes.

Several studies have implicated a role for the ER in the localization of RNAs and, in turn, for localized protein secretion during oogenesis. In *Xenopus laevis*, many maternal RNAs are found concentrated in the animal or vegetal pole of the oocyte and early embryo. One well-characterized protein is *Vg1*, which codes for a transforming growth factor-β and is required for mesoderm induction and right-left symmetry of the embryo (Kloc et al. 2001). In early stage oocytes, *Vg1* is initially distributed throughout the cytoplasm but, as

the oocyte matures, it then becomes tightly associated with a crescent-shaped region of ER and then later with a wedge-shaped ER subdomain (Kloc and Etkin 1998). In late stage oocytes, *Vg1* RNA and closely aligned ER vesicles are redistributed to the vegetal cortex. The localization pattern of *Vg1* is dependent on intact microtubules and kinesin II (Betley 2004). The involvement of this molecular motor, previously established as functioning in membrane trafficking, provides additional support for a role of the ER in *Vg1* transport and localization.

Further evidence for the involvement of the ER in *Vg1* RNA localization is suggested by the putative role of *trans*-acting factors that recognize the *cis*-sequence determinants required for RNA localization. The localization of *Vg1* RNA to the vegetal pole requires a 366-nucleotide element located in the 3′ untranslated region (3′UTR) which is recognized by several proteins (Mowry and Melton 1992). One identified protein is Vera/*Vg1* RBP which is found enriched in ER fractions and is able to accommodate the anchoring of *Vg1* RNA to the ER (Mowry 1996). Overall, the available evidence indicates that *Vg1* RNA piggybacks on ER which is transported on microtubules to the vegetal cortex.

In addition to providing the basis for RNA movement, the ER may provide specialized domains for protein synthesis. The transcript for the yeast plasma membrane protein Ist2p is localized to the bud tip and the protein is deposited locally (Juschke et al. 2004). In *Drosophila*, the anterioposterior and dorsoventral axes of the future embryo are specified by the secreted *Gurken*, a transforming growth factor-α-like protein. *Gurken* RNA is transported and localized to ER at the dorsal/anterior corner of the oocyte resulting in localized exocytosis at this site (Herpers and Rabouille 2004). Hence, RNA localization to specific ER subdomains at discrete cellular locations may be an alternative process for targeted secretion.

3 RNA Localization in Plants

Other than RNA sorting in developing seeds, which will be the subject of the remaining sections of this article, there are relatively few reports on the localization of RNAs in plants. The interested reader is referred to two recent reviews (Okita and Choi 2002; Crofts et al. 2004) which describe published examples of this phenomenon in higher plants and algae. One study that deserves mention is the localization of expansin RNAs. Expansins are extracellular proteins that are involved in cell enlargement, pollen tube growth, and abscission as a result of their ability to induce cell wall relaxation and extension (Cosgrove 2000). Im et al. (2000) showed that the different expansin mRNAs were evident as distinct patches located at either the apical or basipetal end of xylem precursor cells. More specifically, *ZeEXP1* and *ZeExp3*

mRNAs were found at the apical end just below the apical meristem, whereas *ZeExp2* mRNA was found at the basal end. The nature of the RNA patch was not identified, but given the secretory nature of expansins, the "patch" is likely to be a complex of ER membranes. Hence, the targeting of expansin RNAs to a specific subdomain of ER located at either the apical or basal end of the protoxylem cells may function to direct polarized trafficking of the secreted expansin to specific cell wall regions to allow intrusive cell growth.

4
Prolamine and Glutelin RNA Localization in Rice

Plant seeds accumulate storage proteins which serve as a source of nitrogen and carbon for the developing seedling. The storage proteins are coded by two major gene superfamilies, prolamin and globulin (Shewry 1995; Shewry et al. 1995). Unlike other plants, which utilize only one of these storage protein types as their dominant reserve, rice is unique in that it accumulates major quantities of both storage protein types. In addition to accumulating the alcohol-soluble prolamine, the typical storage reserve of cereals, rice contains vast quantities of glutelin, a protein structurally homologous to the 11S globulins. Rice also accumulates significant quantities of a 26-kDa α-globulin. Although the solubility properties of this protein have led it to be defined as globulin-like, it also contains peptide domains of the prolamine superfamily and is related to the rice prolamines. These rice storage proteins are deposited as separate entities within different compartments of the endomembrane system, either the vacuole or ER lumen (Fig. 1). Interestingly, oat also utilizes both storage protein types but they are stored together in a single compartment, the protein storage vacuole (Muench and Okita 1997).

Information on how these proteins are deposited within the ER and protein storage vacuole is rather limited. In view of their close structural identity to the 11S globulins, it is likely that many of the cellular processes involved in the synthesis, transport, and deposition of glutelins are identical to those observed for the 11S globulins (Robinson and Hinz 1999). The newly synthesized protein is exported to the Golgi where it forms dense vesicles which then fuse to the storage vacuole (Krishnan et al. 1986). Protein export and/or dense vesicle formation is likely to depend on the presence of vacuolar peptide sorting signals, as demonstrated for phaseolin (Frigerio et al. 1998). One or more peptide sorting signals are recognized by a receptor located at the transitional ER or an early Golgi compartment, which concentrates glutelin into a dense vesicle that has been detected in developing rice endosperm (Krishnan et al. 1986). The 26-kDa globulin has also been observed to form Golgi-associated dense vesicles (Krishnan et al. 1992), suggesting that they are transported to the storage vacuole by a similar if not identical process.

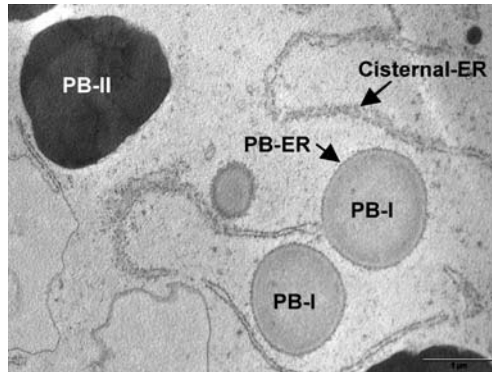

Fig. 1 Morphology of protein bodies in rice. Electron micrograph of a rice endosperm section. Depicted are spherical prolamine-containing protein bodies (PB-I) bounded by rough ER (PB-ER), irregularly shaped glutelin-containing protein bodies (PB-II), and cisternal ER. *Scale bar* = 1 μm

The accumulation of prolamine within the ER lumen could also be dependent on a receptor-based mechanism involving a lumenal peptide sorting signal, although none has ever been identified in storage proteins that utilize this site for storage. Assembly and accumulation within the ER lumen is more likely a product of protein–protein interactions, both homotypic and heterotypic (Kim et al. 2002). For example, the maize β- and γ-zeins are capable of self-assembling into stable lumenal granules in transgenic plants, whereas the α- and δ-zeins are unable to do so and are dependent on interaction with the former pair of zeins for ER retention (Herman and Larkins 1999). This assembly of prolamins into an intracisternal inclusion granule must occur at a rate much faster than its potential export from the ER. Moreover, the inclusion granules must be highly ordered structurally to prevent an unfolded protein response leading to their degradation.

Receptor-dependent and -independent protein interactions are likely responsible for the distinct protein bodies formed in rice. One question that is immediately raised here is how are heterotypic interactions between newly synthesized prolamine and glutelin polypeptides prevented from occurring? One possibility is that these proteins are rapidly sorted from each other; for example, glutelins may be rapidly exported at the transitional ER sites. Alternatively, they are synthesized and initially deposited at spatially distinct subdomains of the ER membrane complex. Indeed, rice endosperm cells contain two morphologically distinct ER subdomains; cisternal ER consisting of single lamellar membranes distributed throughout the cell and protein body ER (PB-ER) which delimits the prolamine protein body (PB-I) (Krishnan et al. 1986; Li et al. 1993). Both membrane types are continuous and constitute the cortical ER in developing rice endosperm (Fig. 1) (Muench et al. 2000).

These distinct ER subdomains may serve to spatially separate the synthesis and, in turn, the entry of these proteins into the secretory pathway. Such a suggestion was supported by biochemical analysis of subcellular fractions enriched for these membranes. Prolamine PBs, purified by repeated sucrose density gradient centrifugation, contained polyA-RNA which directed the synthesis of only prolamines, suggesting that the PB-ERs were enriched for prolamine mRNAs (Yamagata et al. 1986). On the other hand, Kim et al. (1993) demonstrated an approximately two-fold greater abundance of glutelin transcripts over prolamine transcripts in membrane-bound polysome fractions, indicating that this fraction, composed mainly of cisternal ER, is enriched for glutelin mRNAs.

The segregation of these storage protein RNAs to distinct ER subdomains was unequivocally demonstrated by Li et al. (1993). Estimation of the storage protein content in purified PB-I and cisternal ER fractions and in thin tissue sections using high-resolution in situ hybridization techniques showed that the cisternal ER contained more than a twofold molar excess of glutelin mRNA over prolamine transcripts, while prolamine mRNAs were present at seven- to ten-fold molar excess over glutelin transcripts in the PB-ER (Li et al. 1993). The results of this study demonstrated that the storage protein RNAs were not randomly distributed on the ER but, instead, localized to specific subdomains of this membrane complex. Although not directly demonstrated, these results indicate that the storage proteins gain entry into the secretory pathway at spatially separated sites within the ER.

The segregation of these storage protein RNAs to distinct ER subdomains could occur directly by the directed targeting of the RNAs. Alternatively, the localization of the rice storage protein RNAs to distinct ER subdomains could be accomplished by a peptide-based mechanism. Under this scenario, emergence of the signal peptide during translation would be recognized by a signal recognition particle (SRP), which would arrest translation and mediate interaction with the SRP receptor and Sec61p complex on the ER. Distinct SRP–receptor combinations or additional factors would dictate the specificity towards the PB-ER or cisternal-ER membranes. Interestingly, despite the fact that the signal peptides of rice prolamines share considerable homology with those from the maize zeins, which are also deposited as intracisternal inclusion granules, the mature primary sequences of these proteins are unrelated (Masumura et al. 1990; Mitsukawa and Tanaka 1991). The glutelin signal peptide has no sequence similarity to these prolamine sequences and, like other 11S globulin signal peptides, contains two cysteine residues interdigitated by five leucines. Interestingly, the rice genome contains two SRP 54 polypeptides, the subunit of the SRP complex which recognizes the signal peptides (*Oryza sativa* genome database), suggesting the possibility of unique SRPs that recognize distinct signal peptide sequences. Despite these features, the available evidence indicates that prolamine RNAs are localized to the PB-ER by an RNA-based mechanism.

5
Rice and Maize Prolamine RNAs are Localized by an RNA-Dependent Mechanism

The high-resolution in situ hybridization approach, although sensitive, was extremely laborious and alternative more facile approaches were clearly required to study how these RNAs were sorted to the PB-ER and cisternal ER. Choi et al. (2000) developed a light microscopy-based approach using an in situ reverse transcriptase-mediated polymerase chain reaction (RT-PCR) with fluorescently labeled nucleotides to study RNA localization in developing rice endosperm cells. Figure 2 shows that fluorescence patterns obtained by in situ RT-PCR and their spatial relationships to the PB-ER are markedly distinct for prolamine and glutelin RNAs. Prolamine RNAs are distributed as small, spherical, and sometimes ringlike structures of 1–2 μm diameter which are identical to the structures labeled by BiP antibodies and visualized by indirect immunofluorescence (Choi et al. 2000). Consistent with this view is that these structures were also stained by $DiOC_6$ and rhodamine B hexyl ester, two ER vital stains which specifically label PB-I because they also react with the hydrophobic prolamine polypeptides as well as the ER membrane (Choi et al. 2000) (Fig. 2). In contrast, glutelin RNAs are seen as much larger irregularly shaped patches located adjacent to PB-I, a pattern indicative of their localization on cisternal membranes (Fig. 2).

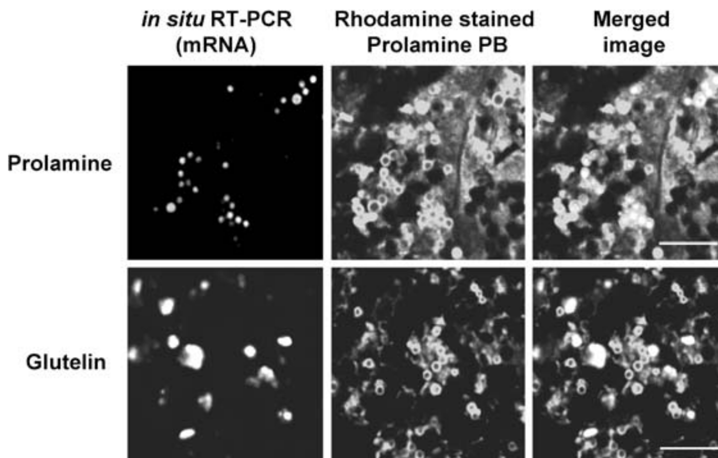

Fig. 2 Prolamine and glutelin mRNA localization as viewed by in situ RT-PCR. Prolamine and glutelin RNAs were detected by in situ RT-PCR using gene-specific primers and Oregon Green 488 d-UTP. The spherical PB-ERs were visualized by staining the sections with Rhodamine B hexyl ester (*middle images*). Confocal microscopic analysis showed that prolamine RNAs were localized to spherical PB-ERs. In contrast, glutelin RNAs were distributed as large patches indicative of their location on cisternal ER located near PB-ER. *Scale bar* = 10 μm

The development of facile methods to assess RNA localization enabled a systematic study to determine whether this phenomenon was dependent on either an RNA or peptide-based elements. A series of transgenic plants expressing synthetic prolamine RNAs were generated and analyzed for RNA localization. They included a wild-type prolamine gene and three prolamine variants containing a substituted glutelin 3′UTR in place of the normal prolamine 3′UTR, a signal peptide deleted version, and an AUG translational knockout. These various prolamine transgenes were also tagged with unique DNA sequences to distinguish the transcripts made from the transgene from those derived from endogenous genes. The signal peptide deleted prolamine RNA was faithfully localized to the PB-ER, indicating that the signal peptide and the corresponding coding sequences were not required. In contrast, the prolamine glutelin 3′UTR RNA was mistargeted to the cisternal ER. As the epitope-tagged prolamine coded by this prolamine RNA variant was readily detected by immunoblot analysis, these results indicate that prolamine RNA localization is not dependent on the prolamine primary sequence. Interestingly, although the prolamine primary amino acid sequence is not essential for RNA targeting to the PB-ER, an intact AUG translation initiation codon is required for correct prolamine RNA localization to the PB-ER (Choi et al. 2000; Hamada et al. 2003b). This result indicates that the regulated prolamine RNA pathway requires the participation of the translational machinery. On the surface, this latter conclusion would appear to conflict with our earlier interpretation of RNA-directed localization. However, the requirement for translation and RNA targeting are not strictly coupled and can be contributed by different sequences, as demonstrated by the analysis of RNA reporters containing translatable green fluorescent protein (GFP) or β-glucuronidase (GUS) sequences. Placement of prolamine sequences in the 3′UTR was able to direct the reporter RNAs to the PB-ER. Consequently, the requirement for translation is provided by the GUS or GFP reporter, with the RNA *cis* elements conferring PB-ER targeting located in the 3′UTR (Choi et al. 2000).

The dependence on RNA localization for protein localization appeared to be a unique feature of the rice system, even though other cereals, e.g., maize and sorghum, accumulate storage proteins in similar compartments to those used by rice. Indeed, based on in situ hybridization analysis using endosperm thin sections, RNAs for the maize zeins were reported to be essentially randomly distributed on the PB-ER and cisternal-ER membranes in developing maize endosperm (Kim et al. 2002). Protein body formation in maize was suggested to occur by diffusion of the zeins to the intracisternal inclusion granule followed by self- and interassembly of the various zein polypeptide classes. Curiously, heterologous expression of the 10-kDa δ-zein RNA in developing rice endosperm showed a striking localization pattern. Unlike the random distribution of the 10-kDa δ-zein RNA reported in maize endosperm, the localization of this RNA was clearly restricted to the PB-ER (Hamada et al. 2003b).

To resolve this apparent discrepancy, Washida et al. (2004) assessed the distribution of the storage protein RNAs in developing maize endosperm using the in situ RT-PCR technique mentioned earlier. In developing maize endosperm cells, the PB-ER was visualized and the localization was confirmed by both double immunofluorescence using 10-kDa δ-zein antibody and rhodamine B hexyl ester staining. Analysis of the mRNA localization using in situ RT-PCR revealed that maize zein RNAs coding for 22-kDa α-zein, 15-kDa β-zein, 27-kDa γ-zein, and 10-kDa δ-zein were localized to ER-bounded zein protein bodies (Washida et al. 2004). In contrast, RNAs coding for 51-kDa legumin-1, a storage protein sharing homology to the 11S globulins and rice glutelin, were distributed on adjacent cisternal ER. These results indicate that the same RNA targeting mechanism is at work in rice and maize, and that this mechanism has been conserved since the divergence of these species 50 million years ago.

The apparent differences in RNA distribution patterns depending on the technique used deserve comment. The in situ RT-PCR technique is significantly more sensitive as the tissue sample is much thicker, resulting in a larger target size as compared to the ultrathin sections of tissue analyzed by in situ hybridization at the electron microscopy level. The basis for the apparent random localization of zein RNAs when viewed by electron microscopy (Kim et al. 2002) is not known. However, the measured gold particle densities in this study were about an order of magnitude lower than those measured in rice (Li et al. 1993). One possibility that may account for the observed random distribution of RNAs was a preferential loss of RNAs associated with the PB-ER during the in situ hybridization procedure, as suggested by Kim et al. (2002).

6
Prolamine RNA Transport from the Nucleus to the PB-ER

RNA transport is well characterized in several systems (Bor and Davis 2004). In polarized somatic cells and during early vertebrate development, endogenous RNAs are visualized as large granules (Barbarse et al. 1995; Ainger et al. 1997; Carson et al. 1998; Roock et al. 2000; Krichevsky and Kosik 2001) or particles (Sundell and Singer 1990; Ferrandon et al. 1994; Forristall et al. 1995; Kloc and Etkin 1995) that move at rates of up to 4 to 6 μm/min via cytoskeleton-associated motors (Bassell and Singer 1997; Chartrand et al. 2001; Saxton 2001; Kloc et al. 2002; Tekotte and Davis 2002). RNA movement can be monitored in real time in living cells using a method based on a modification of the yeast three-hybrid system developed to detect RNA binding proteins (Bertrand et al. 1998; Takizawa and Vale 2000). In this system, two genes are expressed to monitor RNA transport. One gene encodes GFP fused to the MS2 coat protein, a high-affinity RNA binding protein from the single-

stranded MS2 RNA phage which recognizes a specific 19-nucleotide RNA stem loop. This reporter protein also contains a nuclear localization signal (NLS) to restrict it to the nucleus in the absence of any RNA target. A second gene transcribes a hybrid RNA containing prolamine RNA sequence and tandem repeated (6×) MS2 RNA binding sites fused to a GUS reporter gene. When these two genes are expressed simultaneously, the MS2-GFP binds to one or more of the MS2 binding sites, which enables one to follow the movement of this RNA in real time by following the GFP fluorescence.

Hamada et al. (2003a) adapted this GFP two-gene monitoring system to follow prolamine RNA movement in developing rice endosperm and heterologously in tobacco BY-2 cells. When the MS2-GFP fusion protein was expressed by itself in BY-2 cells, native fluorescence was observed predominantly within the nucleus. When coexpressed with GUS-MS2-prolamine RNA, GFP was localized to the periphery of the cell, the presence of the large vacuole in these cells apparently restricting GFP fluorescence to the thin cytoplasm adjacent to the cell wall (Hamada et al. 2003a). A similar pattern was also evident in developing rice endosperm cells when MS2-GFP fusion protein was expressed alone. However, an entirely different distribution pattern was observed when both genes were expressed. Numerous small particles ranging in size from 0.3 to 2 μm in diameter were readily evident. Although many of the particles were stationary, several moved within the focal plane across the cell demonstrating RNA movement. These prolamine transport particles generally moved unidirectionally in a stop-and-go manner, with an estimated average velocity of 0.3–0.4 μm/s and instantaneous velocities of up to 10 μm/s (Hamada et al. 2003a). These characteristics are indicative of movement driven by a cytoskeleton-associated motor protein (Bassell et al. 1999; Jansen 1999, 2001; Tekotte and Davis 2002).

The dependence on the cytoskeleton for RNA movement was supported by the use of drugs that disrupt this cellular structure. Under optimal conditions, RNA movement particles were easily observed for up to 30 min. When treated with cytochalasin D and latrunculin B, which disrupt actin filaments, particle movement was rapidly suppressed with only Brownian movement noted. Movement was also arrested by 2,3-butanedione monoxime, an inhibitor of the ATPase activity of skeletal myosin (Ostap 2002). In contrast, the microtubule drug nocodazole had no significant effect on RNA movement (Hamada et al. 2003a). The results indicate that RNA particle movement is dependent on intact microfilaments.

7 The Dependence of RNA Transport on Zip Codes

The *cis* elements or zip codes (Singer 1993) responsible for prolamine RNA targeting to the PB-ER were defined by studying the localization of RNAs

coded by a series of GFP-prolamine cDNA genes in transgenic rice endosperm cells (Hamada et al. 2003b). GFP RNAs, which normally are localized to the cisternal ER, are redirected to the PB-ER when prolamine RNA sequences are attached to the 3′UTR. Deletion analysis of the prolamine sequences showed that two zip codes are required for PB-ER targeting. One zip code is located downstream adjacent to the sequence encoding the signal peptides, while a second *cis* element is located in the proximal half of the 3′UTR (Hamada et al. 2003b). Both *cis* elements share considerable homology, suggesting that recognition by *trans*-acting factors is sequence dependent.

The majority of *cis* elements responsible for RNA targeting do not share a common sequence, but are dependent on the formation of an exact secondary and/or tertiary structure. This is supported by ongoing studies to identify the *cis* elements responsible for the targeting of the 10-kDa δ-zein RNA to the PB-ER in developing rice endosperm. Despite the relatively small size of this transcript, the RNA appears to have at least four *cis* elements. Three are located within the proximal two-thirds of the coding sequence including the signal peptide and a fourth is located in the 3′UTR (Washida et al. unpublished). A direct alignment of the RNA sequences of these four regions indicates very little sequence homology and no common motifs are evident. Moreover, there is no sequence similarity between the *cis* element of rice prolamine (Hamada et al. 2003b) and those of the 10-kDa δ-zein. This lack of sequence conservation between these RNAs suggests that the *cis* element responsible for PB-ER targeting is structural in nature and that its sequence is less important.

Both the rice prolamine and maize 10-kDa δ-zein require at least two *cis* elements for restricted localization to the PB-ER. These multiple, functionally redundant *cis* elements may act synergistically to ensure efficient transport. Other examples of mRNAs with multiple redundant *cis* elements are found in *Xenopus* oocytes (*Vg1*: Desher et al. 1998; Mowry and Cote 1999; Yaniv and Yisraeli 2001; *Fatvg*: Chan et al. 1999), *Drosophila* oocytes (*Nanos*: Bergsten et al. 2001), and budding yeast (*Ash1*: Gonzalez et al. 1999; Chartrand et al. 1999). In many cases, the location of these multiple *cis* elements is restricted to the 3′UTR. The presence of one or more *cis* elements in the coding regions of the rice prolamine and maize 10-kDa δ-zein may confer another function in addition to their role in RNA localization. The yeast *Ash1* RNA has four *cis* elements, three of which are located within the coding region. Chartrand et al. (2002) obtained evidence that these three *cis* elements are required for transport and localization of *Ash1* RNA to the bud tip, and for preventing premature synthesis of the protein during its transport to this location. If the rice and maize *cis* elements also play a similar dual function, this would be consistent with the requirement for translation initiation where rice prolamine RNAs are transported in translationally arrested complexes to the PB-ER.

8 Multiple RNA Transport Pathways to the Cortical ER

The presence of a single rice prolamine or maize 10-kDa δ – zein *cis* element results in localization of the RNA not only to the PB-ER but also to the cisternal ER. In the absence of any *cis* element, RNAs are localized to the cisternal ER. These results support the existence of a default RNA transport to the cisternal ER in addition to a regulated transport pathway to the PB-ER. RNA transport to the ER occurs even for RNAs that code for cytoplasmic-localized proteins (Hamada et al. 2003a), indicating that the bulk, if not all, of protein synthesis occurs near or on the ER.

Although glutelin RNAs are localized to the cisternal ER, their transport and targeting to this ER subdomain also occurs by a regulated pathway. Replacement of the prolamine or 10-kDa δ-zein 3′UTR with the glutelin 3′UTR results in their redirection from the PB-ER to the cisternal ER. These results suggest the presence of one or more *cis* elements in the glutelin 3′UTR, and the existence of a regulated glutelin pathway to the cisternal ER which is dominant over the regulated prolamine pathway to the PB-ER (Choi et al. 2000; Hamada et al. 2003b).

9 Mistargeting of RNA to ER Subdomains Affects Protein Localization

The sorting of rice storage protein RNAs to distinct ER subdomains, and the packaging of these proteins into separate endomembrane compartments, suggests that RNA localization and protein localization are interrelated. Such a relationship is obvious for prolamine RNAs and the immediate assembly of the coded protein product upon translocation into the lumen. Additionally, proteins synthesized on the cisternal ER would presumably be in close proximity to the transitional ER, a requirement for their export to the Golgi complex or storage vacuole. Because storage protein RNA localization requires specific *cis* elements, this hypothesis relating RNA and protein localization can be directly tested. A hybrid 10-kDa δ-zein RNA containing a 3′UTR from the nopaline synthase (NOS) gene is normally localized to the PB-ER in developing rice endosperm as mentioned before. Immunocytochemical studies of ultrathin sections and transmission electron microscopic analysis showed that 10-kDa δ-zein polypeptides are localized to PB-I, the site of their synthesis (Fig. 3). Replacement of the 3′NOS sequence with the glutelin 3′UTR results in the displacement of the RNA from the PB-ER to cisternal ER (Hamada et al. 2003b). This change in RNA localization is mediated by the *cis* element(s) contained within the glutelin 3′UTR, and takes place even though the 10-kDa δ-zein coding sequence contains three *cis* elements that direct PB-ER localization of the RNA. Further analysis has shown that this altered RNA

localization pattern also changes the localization pattern of the 10-kDa δ-zein protein. The 10-kDa δ-zein was not found in PB-I but in the storage vacuole PB-II (Fig. 3). The final destination of the 10-kDa δ-zein protein therefore depends on which specific ER subdomain is used as the initial site of protein synthesis.

Several questions that arise from this study center on the mechanism by which the 10-kDa δ-zein protein, which is normally deposited as part of an intracisternal inclusion granule, is transported from the cisternal ER to PB-II. One property of the 10-kDa δ-zein is that it appears to be incapable of forming a protein body-like structure by itself in transgenic plants. Lending and Larkins (1989) have demonstrated that the synthesis of the various zein classes shows a strict temporal and spatial pattern during endosperm development, with the β- and γ-zeins laid down first followed by the α- and δ-zeins, which displace the former zein classes to the peripheral regions of the protein bodies. Coexpression of γ- or β-zein is needed for stable α- and δ-zein accumulation and aggregation to form protein bodies in transgenic tobacco (Bagga et al. 1995; Coleman et al. 1996; Bagga et al. 1997; Kim et al. 2002; Coleman et al. 2004), suggesting that the 10-kDa δ-zein (as well as α-zeins) is incapable of self-assembly in a larger macromolecular structure and requires

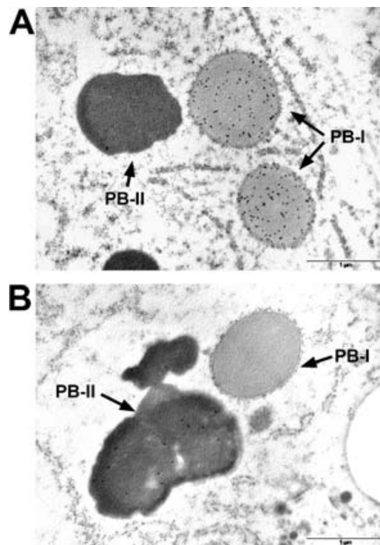

Fig. 3 Protein-A gold immunocytochemistry showing the localization of 10-kDa δ-zein in distinct protein bodies. The 10-kDa δ-zein and hybrid zein–glutelin constructs were introduced into rice by *Agrobacterium*-mediated transformation. In situ RT-PCR analysis revealed that 10-kDa δ-zein RNAs were localized only to the PB-ER, whereas the hybrid zein–glutelin RNAs were targeted to cisternal ER (Hamada et al. 2003b). δ-Zein polypeptides coded by RNAs containing the NOS 3′UTR are observed in PB-I (**a**) , while those coded by RNAs containing the glutelin 3′UTR are localized to PB-II (**b**). *Scale bar* = 1 μm

heterotypic interactions with other proteins. In the absence of interacting partners, the 10-kDa δ-zein is likely to be competent for ER export and transport to the storage vacuole.

A second question relates to how 10-kDa δ-zein is targeted to the storage vacuole PB-II. In general, the mechanism by which storage proteins are sorted to the storage vacuole is poorly understood. Both 7S and 11S globulins are exported from the ER where they are concentrated into dense vesicles at the *cis*-Golgi (Hillmer et al. 2001). Only phaseolin, a 7S globulin, has been shown to possess a vacuolar targeting signal at its C-terminus (Frigerio et al. 1998), although such targeting signals have also been suggested for the 11S globulins (Saalbach et al. 1991). The presence of a vacuolar sorting signal indicates the existence of a receptor that is responsible for ER export and dense vesicle formation. The 10-kDa δ-zein polypeptides may have a cryptic peptide signal that targets them to the storage vacuoles or they may be escorted to this organelle by their heterotypic interaction with glutelins. Immunocytochemical studies showed that the 10-kDa δ-zeins are embedded in the crystalline (glutelin-containing) parts of PB-II, suggesting the possible interaction of these proteins. Conversely, transport to the storage vacuole in rice may be simply a default process. Further studies on protein transport to the storage vacuole in rice should provide information on the existence of any of these pathways.

The preceding description clearly demonstrates that at least for one protein, the 10-kDa δ-zein, the localization of its RNA has a profound impact on where the protein is deposited in the cell. The converse experiment to support the relationship between RNA localization and protein localization is to mistarget an RNA from the cisternal ER to the PB-ER. The sunflower seed 2S albumin (SSA) is stored in the protein storage vacuole in both sunflower and transgenic rice. Closer examination showed that SSA protein is localized on the periphery of PB-II (Washida et al. unpublished) much like the 26-kDa globulin (Krishnan et al. 1986). Consistent with this location in the storage vacuole, its RNA is observed on the cisternal ER much like that seen for glutelin RNAs. Targeting of SSA RNA to the cisternal ER suggests the presence of *cis* elements functionally equivalent to those present in glutelin RNA (Hamada et al. 2003b)

The SSA RNA was modified to contain the 5′ coding sequences of the 10-kDa δ-zein RNA, which contains one or two PB-ER *cis* elements. In both instances, a portion of the hybrid 10-kDa δ-zein–SSA RNA was found to be partially mislocalized to the PB-ER in addition to the remainder which was targeted to its normal location on the cisternal ER. Cytologic examination using SSA antibodies showed that the SSA protein was found in both PB-I and PB-II (Washida et al. unpublished). Although SSA RNA could not be totally displaced to the PB-ER, the results do support the hypothesis that the PB-ER delimits a unique ER subdomain for the confined localization of proteins.

In PB-I, SSA was distributed to the periphery of the inclusion granule. Such a spatial arrangement is similar to that seen for the zein protein bodies, where

the β- and γ-zeins are displaced to the periphery around the α- and δ-zeins. The peripheral distribution pattern also indicates that SSA does not interact with prolamines and self-assembles around the more hydrophobic prolamine inclusion granule. The physical processes that account for the self-assembly of SSA within PB-I are not known, but it is likely that they are identical to those used for the concentration of SSA in Golgi-derived dense vesicles.

10 Conclusion

Recent studies have demonstrated that the localized secretion of proteins is accomplished by targeting their RNAs to ER located near the site of secretion. Ongoing studies in developing rice endosperm have demonstrated that RNA localization to specific ER subdomains is also responsible for the final destination of the encoded protein in the endomembrane system. Three RNA transport pathways have been identified: two regulated pathways requiring targeting *cis* elements and a third default pathway. Current efforts are focused on the characterization of the *trans*-acting RNA binding proteins and their interacting partners, which allow multiple RNA localization pathways from the nucleus to the distinct subdomains of the cortical ER. These molecular cellular studies are being complemented by parallel studies on genetic mutants that contain abnormal amounts of the 57-kDa glutelin precursor, which is normally processed into basic and acidic subunits in the protein vacuole (Kumamaru et al. 1988). Several of these mutants have been identified as RNA sorting mutants, in addition to mutants defective in proglutelin transport to the storage vacuole and proteolytic processing. This combined multifaceted approach will hopefully identify the major players responsible for formation of the RNA particle, and its transport and localization to specific subdomains of the cortical ER.

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